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13. ABSTRACT *(Maximum 200 words)*

Gene targeting in mouse embryonic stem cells was used to generate animal models for the human diseases NF1 and NF2. Chimeric mice composed in part of *Nf1*-/- cells develop neurofibroma-like lesions that contain cells with Schwannian and perineurial features, which are *Nf1*-deficient. This model supports the view that neurofibromas develop in response to full loss of *NF1* function, and indicate that the target cell may be a neural crest derived precursor to the Schwann cell. A second model associated with *Nf1* mutation has been developed through the introduction of a linked mutation in the *p53* gene. These animals rapidly develop tumors, a high percentage of which show features of malignant peripheral nerve sheath tumors (MPNSTs). Importantly, human NF1 patients also develop MPNSTs at high frequency, and these tumors often carry *p53* mutations as well. The Nf/*p53* mutant mice are being studied for possible genetic modifier effects through crossing to other mouse strains. Similar experiments are ongoing with mice mutant for *Nf2*/*p53* and *Nf1*/*Nf2*. Cell-based experiments have revealed increased growth factor sensitivity and reduced cell death in *Nf1*-/- mouse embryo fibroblasts (MEFs), and increased motility and metastatic potential in *Nf2* mutant cells. Studies on the biochemical modification of the *Nf2*-encoded merlin protein indicate a link to Rac1-mediated signal transduction.

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FOREWORD

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Introduction

Neurofibromatosis type I and II (NF1 and NF2) are human genetic diseases affecting the nervous system. In both diseases, individuals inherit a loss-of-function mutation in a tumor suppressor gene and are thereby predisposed to the development of a characteristic set of lesions. For NF1, the most common symptoms are cutaneous and plexiform neurofibromas of the peripheral nervous system, skin pigmentation defects known as cafe-au-lait macules, and benign hamartomas of the iris. NF1 patients are also at increased risk for developing certain malignancies, including neurofibrosarcomas, optic gliomas, myeloid leukemia and pheochromocytoma. In contrast to NF1, NF2 generally affects cells of central nervous system. NF2 patients develop lesions that are sometimes classified as neurofibromas (although these are usually composed exclusively of Schwann cells, in contrast to the multicellular nature of neurofibromas in NF1), but the major symptoms of the disease are schwannomas affecting the eighth cranial nerves, meningiomas and ependymomas. NF2 patients also frequently exhibit posterior subcapsular cataracts. Although not demonstrated experimentally in every case, it is believed that the development of these lesions in both NF1 and NF2 is dependent on the somatic mutation of the remaining wild-type allele of the relevant tumor suppressor gene.

The genes responsible for NF1 and NF2 were cloned in 1990 and 1993, respectively (1-5). The *NF1* gene, located on chromosome 17, encodes a 2818 amino acid protein (neurofibromin) which contains a domain that is structurally and functionally related to the mammalian p120GAP protein as well as the IRA proteins of Saccharomyces cerevisiae (6,7). The GAP-related domain (GRD) of neurofibromin can stimulate the GTPase activity of mammalian Ras proteins in vitro and can functionally complement IRA mutations in yeast (8-10).

The *NF2* gene shares no structural similarity with NF1. Located on chromosome 22, it encodes a protein (merlin) which belongs to a family of cytoplasmic proteins that are believed to link transmembrane proteins to the cytoskeleton (4,5). Merlin is most similar to a group of three closely-related proteins: ezrin, radixin and moesin (the ERM proteins). The N-terminal half of merlin has 60% amino acid identity with the N-terminal halves of the ERMs, while the C-terminal domains are more divergent. The ERM proteins are thought to be regulated by inter- or intra-molecular association that serves to mask functional domains of the proteins (11,15,16); such interactions have now also been demonstrated for merlin itself (17,18). Dissociation of these interactions by phosphorylation and/or binding of lipid molecules may activate the ERM proteins and merlin to bind simultaneously to the transmembrane proteins (including CD44) through their N-terminal domains and to other targets (including the actin cytoskeleton) through a domain near the C-terminus, thereby affecting cellular morphology and function. Like the ERM proteins, merlin localizes to the cytoplasmic face of the plasma membrane, and is particularly enriched in actin rich areas such as membrane ruffles and filopodia (19,20).

The cloning of the *NF1* and *NF2* genes in the past eight years represents a critical step forward in our understanding of the molecular basis for these diseases. Mutational analysis has strongly indicated that each of these genes belongs to the class of tumor suppressors, which contribute to tumor development when debilitated or lost. As with other genes in this class, *NF2* is also mutated in sporadic tumors of the types associated with the familial disease (as well as in a percentage of sporadic mesotheliomas); *NF1* mutations have been detected in a small number of sporadic tumors also (21-23). As tumor suppressor genes, both *NF1* and *NF2* are thought to regulate proliferation negatively when functioning

normally. Moreover, the fact that both genes encode proteins that have recognizable relatives and functional domains provides a framework from which to understand their normal cell biological function and the effects of their mutation in tumor development. Loss of neurofibromin function clearly alters Ras regulation in some contexts, and merlin's association with the plasma membrane and the actin cytoskeleton would be expected to provide clues to its function as well.

While the progress reviewed above has greatly improved our understanding of the molecular basis of the development of NF1 and NF2, there is still a great deal that is poorly understood or completely elusive. Perhaps primary among these is the nature of the specific growth regulatory pathways in which neurofibromin and merlin act. The characterization of these pathways (along with the defects caused by loss of *NF1* or *NF2* function) will go a long way toward defining the basis of disease pathogenesis and will facilitate the development of novel therapies for these diseases.

The approach of my laboratory to the study of the *NF1* and *NF2* genes and their associated diseases has been to construct mouse strains with targeted mutations in the murine homologues of *NF1* and *NF2*. The initial characterization of strains heterozygous for an *Nf1* or *Nf2* mutation has revealed that these genes do act as classical tumor suppressors in the mouse. However, in both cases, the disease phenotype is distinct from that seen in comparably mutant humans. Specifically, *Nf1*+/- mice develop myeloid leukemia and pheochromocytomas (both of which occur in NF1 patients at increased frequency as well), but they do not develop obvious neurofibromas, Lisch nodules, or pigmentation defects (24-27). Likewise, *Nf2*+/- mice are predisposed to late-onset osteosarcomas and fibrosarcomas, but they do not develop the schwannomas or other CNS tumors that occur in human NF2 (28,29). The generation and initial characterization of these strains was supported by grants from the National Neurofibromatosis Foundation and the Medallion Foundation (NF1) and from a previous grant from the Department of the Army (NF2).

The current Department of the Army grant funds the second-generation studies in the development of animal models for these diseases. In addition, these mutant mouse strains and other reagents developed in the process of these studies have provided us with unique set of tools for addressing the function of the *Nf1*- and *Nf2*-encoded proteins. Thus, another major component of the grant is the characterization of cells deficient for these proteins and the development of structure/function assays for *Nf1* and *Nf2*. Because this grant encompasses two distinct disease models (and various derivatives thereof) and two unrelated proteins, it is relatively broad ranging. However, the historical connection between these two diseases and the extensive interaction among researchers investigating them makes this synthesis a logical one. More importantly, the methodology used in the study of these two models and proteins overlaps extensively, resulting in significant synergy.

Body

This progress report has been divided into sections corresponding to the Technical Objectives/Specific Aims of the original grant, which was initiated on 30 September, 1997.

1. Development of existing mouse model of NF1. We have previously shown that chimeric mice composed in part of *Nf1*-deficient cells (made by injecting *Nf1*-/- ES cells into wild-type blastocysts) develop numerous neurofibroma-like lesions throughout the peripheral nervous system. Over the past year, we have completed our characterization of the cell types present in these lesions by marker analysis and electron microscopy (EM).

Initial marker analysis of the neurofibromas in these so-called "*Nf1* DKO chimeras" had indicated that they did not express the Schwann cell marker S100, in contrast to what has been observed for most human neurofibromas (Fig.1). We have shown more recently that these lesions also fail to stain with antibodies directed at other markers of mature Schwann cell cells, such as myelin basic protein, P0 and the p75 subunit of the nerve growth factor receptor (data not shown). In addition, although initial EM analysis had suggested that the highly abundant cells in these lesions may be perineurial cells, we have not been successful in staining them with the marker the perineurial cell marker EMA (data not shown). Interestingly, however, our recent EM data shows quite clearly that the neurofibromas in *Nf1* DKO chimeras are composed of cells with Schwann cell features, perineurial cells as well as fibroblasts, a pattern typical of neurofibromas in NF1 patients (see Fig. 2). One explanation for the apparent discrepancy between ultrastructural and marker analyses is that homozygosity for the *Nf1* mutation blocks the differentiation of a precursor cell of neural crest origin at a point prior to the expression of the end-stage markers tested above. Consistent with this hypothesis, we have observed expression of nestin, a marker of precursor cells in the neural crest lineage, in a small number of *Nf1* DKO chimera lesions (data not shown). This possibility notwithstanding, the EM data demonstrate that the *Nf1*-deficient cells do appear to have the capacity to differentiate into cells with the features of Schwann cells and other mature cells of the nerve. Following the submission of the initial report describing this model (which is currently in preparation), we will address this question more completely by staining lesions with additional markers of the neural crest lineage as well as to examine the differentiation capacity of *Nf1*-/- neural crest cells in vivo and in vitro.

At the time of submission of this grant, we were not in a position to address another important question regarding the development of the neurofibromas in *Nf1* DKO chimeras: is the growth of these lesions due to a cell-autonomous defect associated with *Nf1* deficiency or are *Nf1*-deficient cells of unknown type acting *in trans* to support the growth of the lesions regardless of the genotype of the proliferative cell. We have attempted to address this question by using *in situ* hybridization against the *neo* and *hygro* mRNAs that we assume will be expressed in the *Nf1*-/- cells (*neo* and *hygro* genes were used in the two rounds of gene targeting to inactivate the *Nf1* genes in the DKO ES cells). However, we have observed considerable background hybridization with these probes, and have, therefore, abandoned this approach. As an alternative, we have generated ES cells that are homozygous for an *Nf1* mutation and also carry a ubiquitously-expressed *LacZ* transgene. These cells were made in a two-step process. First, ES cells with the genotype *Nf1*+/-;Lacz+ were generated *de novo* from blastocyst-stage embryos following mating of the *Nf1* mutant strain with the ROSA-26 strain (which harbors the *LacZ* gene) (30). These *Nf1*+/- cells were then subjected to a second round of *Nf1* gene targeting using a *hygro*-containing vector. Potential clones were cultured in G418 plus hygromycin and screened for loss of the remaining wild-type *Nf1* allele by Southern blotting (data not shown). These ES cells have been injected into C57BL/6 blastocysts, yielding three chimeras to date. In two of these animals, we have detected several peripheral neurofibromas. This result confirms our earlier findings that *Nf1*-deficient cells promote the growth of neurofibromas, and, in principal, the ROSA-26 LacZ transgene should allow us to address the genotype of the cells in these neurofibromas. As shown in Figure 3, in the four lesions that we have tested, all of the hyperproliferative cells appear to stain with X-gal, indicating that all of the these cells are derived from the *Nf1*-mutant ES cells. This result strongly suggests that *Nf1*-deficiency causes a cell autonomous proliferative defect. Our ultrastructural analysis would indicate that these hyperproliferative cells are precursors in the Schwann cell and perineurial cell lineages. We are not able to determine whether *Nf1* deficiency is sufficient

for the development of these lesions or whether other, somatically acquired mutations act in conjunction with the *Nf1* mutation to promote their growth.

2. Structure/function analysis of neurofibromin. One of our long term goals is to place neurofibromin into a known signaling pathway(s) in order to provide a framework for understanding the consequences of its mutation in disease. Our favored approach to this question is to characterize defects in *Nf1*-deficient mouse cells, either in vitro or in vivo, and then attempt to complement those defects using wild-type and mutant alleles of *Nf1*. As described above, over the past year, we have continued our characterization of the *Nf1* DKO chimeras, which will lead to future attempts to inhibit the development of neurofibromas following re-introduction of *Nf1* function using a YAC-based approach. To this end, we obtained a series of YACs that harbor the murine *Nf1* gene from the laboratory of Eric Lander (MIT). Over the past year, we have been modifying these vectors for eventual use in mammalian cells. For example, a puromycin resistance gene has been recombined into the YAC in order to allow selection of ES cells that are already resistant to G418 and hygromycin (data not shown). We have also been collecting a series of additional yeast targeting vectors that will be used to create mutations in different domains of *Nf1*. As a complement to the YAC-based approach, we have also recently obtained a full length human *NF1* cDNA vector from Andre Bernards (MGH). Provided that this vector can be stably maintained in bacteria (many laboratories have experienced difficulty in propagating full length *NF1* cDNA clones in the past) , it could facilitate the reintroduction of wild-type and mutant alleles of *NF1* into *Nf1*-deficient ES cells and mouse embryo fibroblasts (MEFs; see below).

Nf1-/- MEFs can be readily obtained from E13.5 homozygous mutant embryos. Over the past year, we have begun to characterize the growth properties, cell cycle profile and resistance to apoptotic stimuli of these cells compared to wild-type MEFs. As shown in Figure 4, *Nf1-/-* cells have somewhat increases sensitivity to serum and epidermal growth factor relative to controls. The mutant cells are also of smaller overall size and grow to higher saturation density (data not shown). We are continuing to determine the specific growth factor requirements of these cells as well as performing biochemical analysis of the RAS pathway and other intracellular signaling pathways. This basic characterization is a prelude to *Nf1* reconstitution experiments in the near future. Another area of interest that has emerge over the past year is the resistance of these cells to apoptotic stimuli. Vogel et al. have previously shown that *Nf1*-deficient neuronal precursors are less dependent on neurotrophic survival factors compared to wild-type controls (31). We have recently addressed whether this is a general property of *Nf1*-mutant cells by examining the response of *Nf1-/-* MEFs to a number of apoptotic stimuli. Although not all such signals differentially affect these cells, they do appear to be relatively resistant to cell death. One representative experiment is illustrated in Figure 5; here the cells were grown in the absence of serum for two days and extent of apoptosis was measured by trypan blue exclusion and the TUNEL assay (32) . These data (along with those of Vogel et al.) may be explained by the fact that *Nf1*-deficient cells would be expected to have increased signaling through the RAS pathway, which could enhance cell survival through the PI3K/AKT pathway. Alternatively, neurofibromin may be an effector of cell death in response to some apoptotic stimuli, in which case *Nf1*-deficient cells would be intrinsically less responsive to these signals. We are currently examining the levels, biochemical modification, and subcellular localization of neurofibromin following treatment of wild-type MEFs with various conditions associated with cell death. The possibility that neurofibromin might act as a modulator of cell death (as opposed to proliferation) drastically alters one's view of the effect of *NF1* mutation in the development of disease and suggests alternative therapeutic strategies.

3. Screen for genes modifying *Nf1* or *Nf2* in mice. One of the attractive features of studying a disease process in an animal model is the ability to readily manipulate the system in a variety of ways and assess the effect on disease development. For example, over the past two years we have been examining the effects of combined mutations in *Nf1*, *Nf2* and *p53* on tumor development in the mouse. The strategy for generating these strains was outlined in the original grant proposal; of note, all three of these genes are located on mouse chromosome 11. Interestingly, mice heterozygous for germline mutations in any two of these three genes are highly cancer prone. This effect is particularly striking when the mutations are located "*in cis*" (that is, on the same chromosome 11).

As we have reported over the past year, most of the tumors that arise in the *Nf2/p53* mutant mice are osteosarcomas (29). Our original assessment of the *Nf1/p53* "*in cis*" tumors was that they fell into the broad class of fibrosarcomas, some of which had features of muscle differentiation indicative of rhabdomyosarcomas. A subset of these tumors were also found to stain positively for the Schwann cell marker S100. In the past year, we have refined our characterization of the tumors from *Nf1/p53* mice and now believe that a significant subset (at least 50%) are malignant peripheral nerve sheath tumors (MPNSTs). This evaluation is based on the presence of S100 staining, nerve involvement and overall histological appearance (see Figure 6). This finding is of particular importance because it is known that a significant percentage of MPNSTs that develop in NF1 patients have mutations in *p53*. Thus, the *Nf1/p53* mutant strain represents an animal model of the more malignant tumor phenotype associated with human NF1, and these animals will be very valuable in assessing the molecular and histopathological progression of these tumors. They will also be useful is establishing the efficacy of potential therapies directed against *Nf1*-deficient tumors (see below).

Another important application of the *Nf1/p53* mutant strain is to begin to address whether genetic modifiers of *Nf1* can be found in the mouse. The use of the compound mutant strain in this screen, although more complex genetically, is necessitated by the fact that the tumor development in *Nf1*+/- mice occurs with very long latency (26). By contrast, the majority of *Nf1/p53* "*in cis*" mice develop tumors with the first six months of life. Therefore, the timecourse of an experiment (the assessment of the tumor phenotype as a function of genetic background) is considerably reduced. Our interest in *Nf1* modifiers is motivated in part by the fact that genetic background has been suggested to influence the expressivity of human *NF1* mutations. Specifically, studies on families affected by NF1 give evidence for genes besides *NF1* itself modifying the severity of the disease, with monozygotic twins showing less variability in disease presentation than other siblings or more distantly related individuals (33). Although there is no direct evidence for modifiers of the NF2 mutant phenotype in humans, an analogous screen to that described below will be performed for *Nf2/p53* mutant animals. It is hoped that by comparing the relative effects of potential modifier loci on different pairwise combinations of mutations in the mouse, we will be able to assign the effect of the potential modifier loci to *Nf1*, *Nf2*, *p53* or to tumor development more generally.

In order to map modifier loci and ultimately identify the responsible genes, the tumor predisposing mutations had to be bred onto a pure inbred onto pure genetic backgrounds. (Our previous analysis had been performed on a mixed C57BL/6 (B6) x 129/sv background.) At the start of this grant period, the *Nf1* mutation had been backcrossed onto the B6 strain five generations and onto the BALB/c strain for seven generations; the *p53* mutation was available from Jackson Laboratories (Bar Harbor, Maine) on both pure B6 and BALB/c backgrounds. In the past year, we have completed backcrossing the *Nf1*

mutation onto the B6 and BALB/c backgrounds for a total of 10 generations, and the purity of the background has been confirmed by simple sequence length polymorphism (SSLP) testing. Compound *Nf1/p53* heterozygotes "*in trans*" have been constructed on both of these backgrounds, and these mice are being crossed to wild-type animals to generate double heterozygotes "*in cis*." The meiotic recombination event required to place the mutations onto the same chromosome 11 is expected to occur at approximately 5% frequency, based on the genetic distance between the to genes and our previous experience. A small number of offspring from these crosses have been typed to date without any double heterozygotes being identified. Such crosses will continue until sufficient double heterozygotes have been generated. As explained in more detail in the original grant, we will compare the tumor phenotypes of the B6 and BALB/c strains (as a first step in identifying potentially recessive modifiers that might exist between these two strains) and also use these parental strains to breed to a series of other "tester" strains, in order to determine the tumor phenotype in the F1 progeny; this second screen will identify dominantly acting modifier genes.

During the development of the congenic B6 and BALB/c strains, we have been conducting a pilot experiment using a less pure strain. We are currently characterizing the tumor phenotype of *Nf1/p53* "*in cis*" mice on an enriched (backcross generation 6) B6 background and have begun to cross the mutation to seven tester strains (A/J, CBA/J, C3H/HeJ, DBA/2J, LP/J, SJL/J, M. castaneous). To date we have 35 double heterozygous mice enriched for B6 background. Of those, 14 have died or been sacrificed due to tumor burden. Six tumors have been identified as sarcomas (immunohistochemistry is ongoing to determine whether they are in the MPNST class), two as thymic lymphomas, one as a diffuse fibromatosis, and five remain to be identified. The mice thus far have died between the ages of 3.2 and 8.2 months (Table 1). We also have F1 progeny from crosses to all of the tester strains (see Table 1). Of the different crosses, pups from the A/JxB6 and CAST/EixB6 crosses are still too young to determine whether they will show a shift in the tumor phenotype, as compared to B6. F1 animals from the CBA/JxB6, DBA/2JxB6, and LP/JxCB6 crosses have not yet shown any tumors and are at the age when B6 mice begin

Table 1
Analysis of Nf-1-/+;*p53*-/+ cis mice on C57BL/6 and F1 hybrid backgrounds

Strain	# Cis Mice	# WT Sibs	# Dead	Average Age of Death*	Youngest Living Mouse	Oldest Living Mouse
C57BL/6	35	12	14	5.2 mo	1.7 mo	8.1 mo
A/JXB6	9	4	0		2.0 mo	2.9 mo
CASTXB6	8	12	0		0.9 mo	2.9 mo
CBAXB6	14	10	0		2.0 mo	4.7 mo
C3HXB6	26	11	3	4.1 mo	2.6 mo	4.7 mo
DBA/2XB6	6	7	0		1.0 mo	4.7 mo
LP/JXB6	4	1	0		3.7 mo	3.7 mo
SJLXB6	36	10	5	3.8 mo	3.0 mo	4.7 mo

* Average age of death is the average of the mice that have died so far. Because the experiment is still in progress, it is too early to determine the mean age of death. The C57BL/6 average age of death is greater than the C3HXB6 or SJLXB6 strain because the experiment has been in progress longer and the mice are older (compare age of oldest living mouse for different strains).

to develop tumors. If these animals continue to be tumor free, this would indicate that these strains carry resistance genes. Pups from C3H/HeJxB6 and SJL/JxB6 crosses have begun developing tumors, around the age that B6 mice develop tumors. We are in the process of characterizing the tumor phenotypes. If these tumors are of the same type as those seen in the B6 mice, it would suggest that these strains do not carry dominant modifier genes. However, if the tumors are of a different type, it would suggest that these strains carry modifiers of the tumor type. Subsequent characterization, crossing and mapping strategies are outlined in the original grant. Finally, we are collecting DNA and RNA samples from all of these tumors for eventual use in SSLP-based loss of heterozygosity analysis and gene expression analysis.

4. Characterizing *Nf2* deficiency in adult chimeras and MEFs. As in the study of *Nf1*, we have constructed chimeric mice composed in part of *Nf2*-deficient cells. As we have reported previously, *Nf2*-/- cells are capable of contributing to many tissues in chimeric embryos. Of considerable interest, these chimeras exhibit numerous developmental and histological abnormalities, indicative of a requirement for merlin function very broadly in the developing mouse. (This aspect of the *Nf2*-/- phenotype is being studied by my former postdoctoral fellow Andrea McClatchey.) At the time of submission of this grant, we had succeeded in generating a small number of adult chimeras with low contribution of *Nf2*-/- cells. While we still plan to generate more of these animals in order to determine whether some of these animals will develop tumors related to those found in human NF2. However, with the recent development of a conditional allele of *Nf2* (M. Giovannini, personal communication), this aspect of the project has not been given high priority. Instead, we have been focusing on the isolation and characterization of *Nf2*-/- MEFs, in part as a complement to our studies on the biochemistry and cell biology of merlin function (see below).

Our normal procedure for MEF isolation uses embryos at E12-14. However, because *Nf2*-/- embryos fail in development at the time of gastrulation (~E7), we developed an alternative strategy to obtain merlin-deficient cells. Chimeric embryos have been produced with *Nf2*-/- ES cells (which carry a neomycin resistance gene), some of which survive until day 12 of gestation. The standard MEF isolation protocol has been applied to these embryos, with the additional step of selecting the mutant cells with G418. In this way, pure (or highly enriched) cultures of merlin-deficient cells can be isolated for analysis in tissue culture. In addition to determining the growth characteristics, cell cycle profiles and growth factor requirements of these cells, we are interested in other parameters related to adhesion, motility, invasion and other aspects of cytoskeletal regulation. This interest stems from the fact that merlin belongs to a family of proteins that are thought to participate in these processes. Furthermore, we have shown that the tumors that arise in *Nf2*+/- mice frequently metastasize (which is typically rare in the mouse) and *Nf2*-/- tumor cell lines also have increased metastatic potential compared to controls (29). Finally, our biochemical analysis has demonstrated a link between merlin and the Rac1 small GTPase, which has been previously associated with motility, invasion and metastasis (see below). We have only begun to analyze merlin-deficient MEFs, but initial experiments have revealed that they do have an increased rate of motility as determined by a standard wound healing assay of a monolayer of cultured fibroblasts (Fig. 7). The rate of would healing is approximately 2-3 fold faster in the *Nf2*-/- cells. Given the central role of Rac in cell motility and the requirement of Rac for motility in this particular assay, these data are consistent with loss of merlin function resulting in deregulation of Rac-dependent signaling pathways. We are currently examining other aspects of Rac-dependent signaling in these cells.

Subsequent analysis of the signal transduction pathways in which merlin participates will require reintroduction of *Nf2* (and mutant derivatives) into the merlin-deficient cells, both MEFs and tumor-derived cells. We have recently obtained a series of retroviral vectors carrying full-length merlin and other domains from Nancy Ratner (U. Cincinnati). In addition, we have subcloned merlin and various domains into a tetracycline-inducible retroviral expression vector (34) and have been establishing conditions for efficient retroviral production. These conditional expression vectors will be particularly useful if merlin expression proves to be toxic in these cells.

5. Continued investigation of merlin function. Over the past year, we have published two manuscripts on the regulation and subcellular localization of merlin. In the first manuscript, we demonstrated that merlin migrates as a doublet around 70 kDa on SDS-PAGE electrophoresis (35). Using a combination of *in vitro* phosphatase treatment and *in vivo* orthophosphate labeling to detect phosphorylated proteins, we showed that the slower mobility form was hyperphosphorylated with the faster mobility species being un- or severely hypophosphorylated. We and others have also shown that merlin localizes similarly to the related ERM proteins to cortical actin, particularly in membrane ruffles as well as other dynamic actin structures (19). Given this localization and merlin's function as a tumor suppressor, we investigated whether merlin might play a role in mediating growth arrest due to contact inhibition. First, we examined whether merlin protein levels or phosphorylation status are affected by increasing confluency in a number of cell lines. Indeed, as cell density was increased the level of merlin protein increased, with the unphosphorylated species being upregulated particularly near the onset of arrest (35). We also demonstrated that in subconfluent, cycling cells, merlin protein levels could be similarly upregulated by serum deprivation, and that this effect synergized with the effect of confluency (35). Importantly, merlin is not simply arrested by growth arrest per se, nor by other G0/G1 arrest inducing stimuli, arguing for a specific response of merlin to cell density and growth factor microenvironment (35). Finally, we were able to demonstrate that merlin phosphorylation is also controlled by cell matrix attachment, as the protein was rapidly dephosphorylated upon placing cells in suspension, an effect which was reversed upon replating; cell-substratum attachment was also shown to be required for the serum-induced phosphorylation of merlin (35).

In an independent study, we found that the localization and the phosphorylation of the related ERM proteins is controlled by the activity of the RhoA small ras-like GTPase (36). Activated alleles of RhoA were sufficient to cause relocalization of ERM proteins to apical/membrane actin protrusions in fibroblasts and cause serine/threonine phosphorylation of the proteins. Inhibition of Rho activity blocked both the relocalization to growth factors and the phosphorylation of ERM proteins. Another laboratory independently demonstrated that Rho-kinase, which is activated by RhoA, can induce the phosphorylation of a conserved threonine residue (T577) in the ERM proteins in vitro (37). Using a phospho-specific antibody, they demonstrated that this site is phosphorylated *in vivo* in response to growth factors.

Given these results, we have been investigating whether RhoA or a related small GTPase might be responsible for controlling phosphorylation of merlin, which we previously demonstrated was also serine/ threonine phosphorylated (Shaw et al., 1998a). We co-transfected activated or dominant negative alleles of RhoA, as well as the related GTPase Rac1 and examined merlin phosphorylation by the mobility shift on SDS-PAGE. Surprisingly, unlike the ERM proteins, activated RhoA did not alter merlin's phosphorylation status, but two different activated alleles of Rac1 strongly potentiated merlin phosphorylation (Fig. 8). This is a particularly interesting finding because of our previous demonstration that *Nf2-/-* tumor cells have increased metastatic potential (Rac has

been implicated in metastasis independently (38,39), and it suggests a series of experiments to explore the functional significance of this interaction. As a start, we have crudely mapped the site(s) of phosphorylation by co-transfecting deletion mutants of merlin with activated Rac alleles. From this analysis, we found that the Rac-responsive sequence lay in the carboxyl half of the protein and further mapping has suggested that at least one Rac-responsive site exists between residues 476-537 (Fig. 8). Examination of the amino acid sequence in the carboxyl terminus of the protein indicated there were 8 serine or threonine sites with flanking residues matching known kinase consensus recognition motifs. Additionally, we found two more serine or threonine residues that were conserved in Drosophila merlin (40); the equivalent of the threonine residue in the ERM proteins which is a target of Rho-kinase (T577, see above) is also present in merlin. To test whether any of these residues were the target of Rac1-induced phosphorylation, we generated site-directed serine/threonine to alanine substitutions at each of these 11 sites individually. These mutants were then co-transfected with activated Rac1 alleles and merlin protein analyzed by western blotting. As shown in Figure 9, there was no effect on the Rac-dependent phosphorylation in 10 of these alanine mutants, while substitution of serine 518 with alanine completely abolished the ability of Rac to induce the mobility shift of merlin.

In an initial attempt to characterize the effect of the Rac-dependent phosphorylation on merlin, we addressed whether it might regulate merlin's association with the cytoskeleton. Thus, we performed detergent (Triton X-100) extraction analysis on merlin the absence or presence of activated Rac. In serum-starved cells, all merlin protein is found in the detergent insoluble fraction. When activated Rac was co-transfected with merlin, the protein became heavily phosphorylated and a significant fraction of the phosphorylated species shifted to the soluble fraction (data not shown). These data suggest that this phosphorylation event may either inactivate merlin by displacing from its active site on the cytoskeleton, or alternatively, may serve to activate a novel form of the protein which now translocates to the cytoplasm where it mediates some effect of Rac-dependent signal transduction.

6. Therapeutic evaluation of farnesyltransferase inhibitors. A long term goal of the development of animal models of NF1 and NF2 is to use these animals in the evaluation of potential therapies for these diseases. Presently, one class of compounds, the farnesyltransferase inhibitors directed against the RAS signaling pathway, are logical candidates in the treatment of tumors with *NF1* mutations. In time other compounds will be developed with potential utility in the treatment of NF1 or NF2, which can be considered for use in these mice.

Over the past year, we have received a commitment from Merck Pharmaceuticals to supply us with 1 gram of their farnesyltransferase inhibitor L-744,832, which has been used to treat RAS-dependent tumors in mice (41,42). Given the uncertainty in the supply of this compound in the future, we have decided to perform our evaluation on the tumors that arise *Nf1/p53* "*in cis*" mice. Moreover, in order to control the experiment carefully, we are waiting until we have generated B6 congenic mice to perform these experiments. Sufficient numbers of mice should be available in the next three to six months, at which time the trials will be performed as outlined in the original grant. Assuming that we are able to obtain additional amounts of this compound (or others from other sources) we will also evaluate the efficacy in the *Nf1* DKO model.

Conclusions

During the first year of this grant period, we have made considerable progress toward our goals of characterizing and optimizing mouse models of NF1 and NF2 and developing assay systems aimed at elucidating the biochemical and cell biological mechanisms of action of the responsible proteins.

The two NF1 models (*Nf1* DKO chimeras and *Nf1/p53* "*in cis*" mice) will be described in a manuscript that is currently in preparation. Both of these models will be useful in examining the development and progression of the tumors that develop in NF1 patients, using a variety of molecular approaches (e.g., LOH and gene expression analysis) and more traditional immunohistochemistry. Furthermore, we are now in a position to determine whether the tumor phenotype in *Nf1/p53* mutant animals is subject to modifier effects from loci present in other strains. The discovery of such modifier genes will lead to the analysis of the importance of human homologs in NF1, and they will also inform us about the underlying mechanisms of disease development. Over the next year, we will initiate a similar analysis of *Nf2/p53* and *Nf1/Nf2* mutant animals in order to identify modifiers of the *Nf2* mutant phenotype. The development of *Nf2-/-* chimeric adult animals has been a disappointment to date, and we have de-emphasized this project in my laboratory; this decision was strongly influenced by the development of a conditional allele of *Nf2* (as yet unpublished) from another laboratory.

We have also made strides in characterizing the phenotype of *Nf1*-deficient MEFs, and our discovery that they are relatively resistant to certain apoptotic stimuli is particularly exciting. Over the next year, we will attempt to determine how neurofibromin normally participates in cell death pathways and/or how its loss protects against cell death. The characterization of the growth properties and investigation of the effect of *Nf1* mutation on known signaling pathways will continue as well. A critical tool in the investigation of the function of neurofibromin is vector system for expressing the full-length molecule and mutant derivatives. The *Nf1*-containing YAC vectors that we have assembled can be stably propagated in yeast and transferred to mammalian cells; it is also relatively straightforward to mutate YACs by targeting in yeast. The *NF1* cDNA vector that we have received may allow even more straightforward mutational analysis, provided that it can be stably propagated.

Our investigation of the regulation of merlin function by phosphorylation and the connection to Rac-mediated signal transduction pathway have provided some of the first clues into the function of this molecule. These findings fit nicely with our observations that *Nf2*-mutant tumor cells have increased metastatic potential and *Nf2-/-* MEFs have increased motility in a wound healing assay. Considerable more work is required to place merlin more precisely into this pathway and to understand the consequences of *Nf2* mutation in cells in vitro and in tumorigenesis in this context. Here again, the ability to reconstitute merlin function into deficient cells is critical to this effort, and we believe that the retroviral vector systems that we have acquired and assembled over the past year will be invaluable in this regard.

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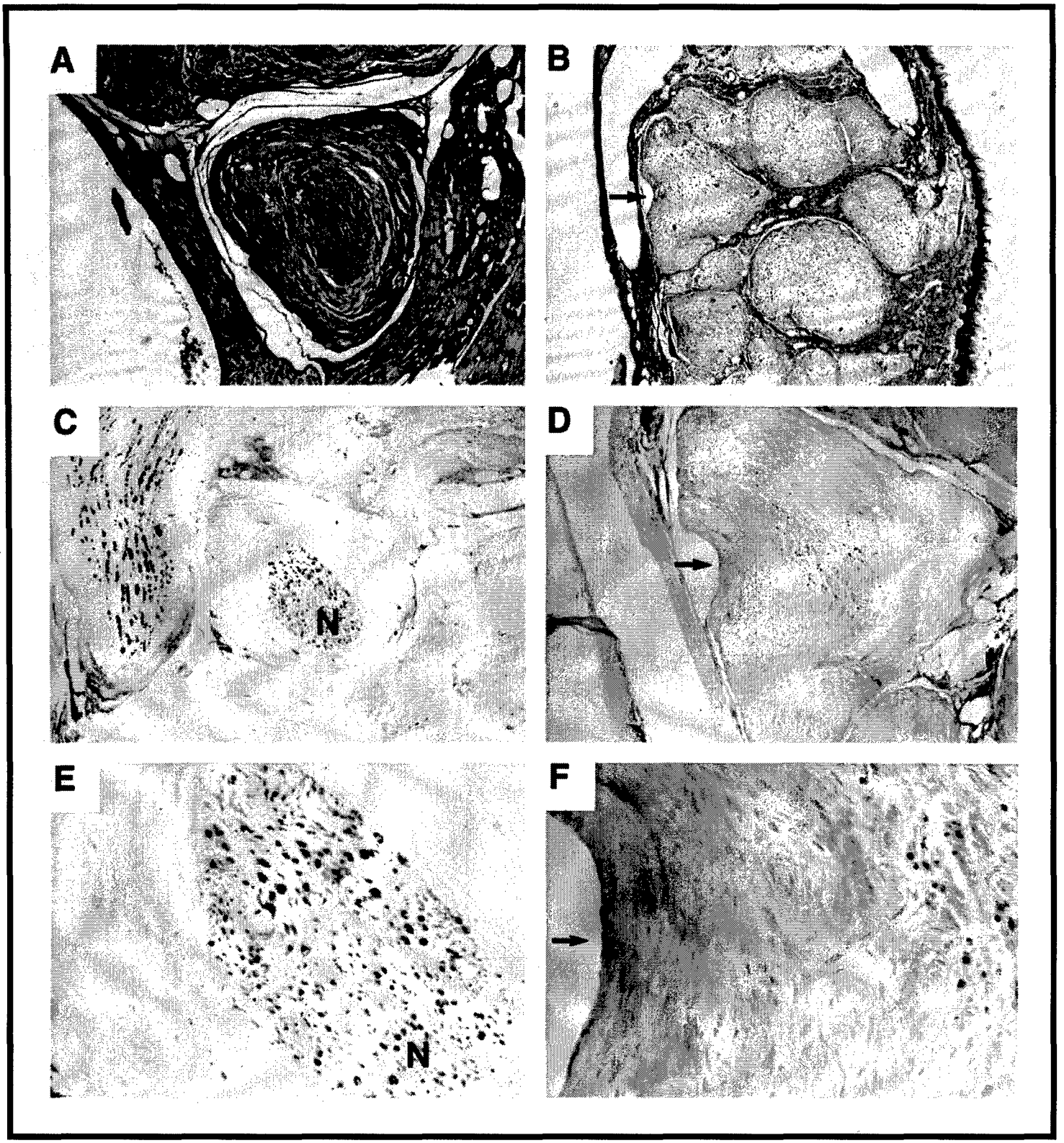

Figure 1. Histological analysis of tumors from Nf1-/- chimeric mice.
A, B) Hematoxylin and eosin stained sections of lesions arising in an animal's back and tongue, respectively. (Note the multinodular nature of the tongue lesion(s)). Only cells associated with the central normal nerve (N) stain positively for S100 in the back lesion (C: 10x, E: 40x) and in a nerve remnant in the tongue (center D: 10x, F: 40x).

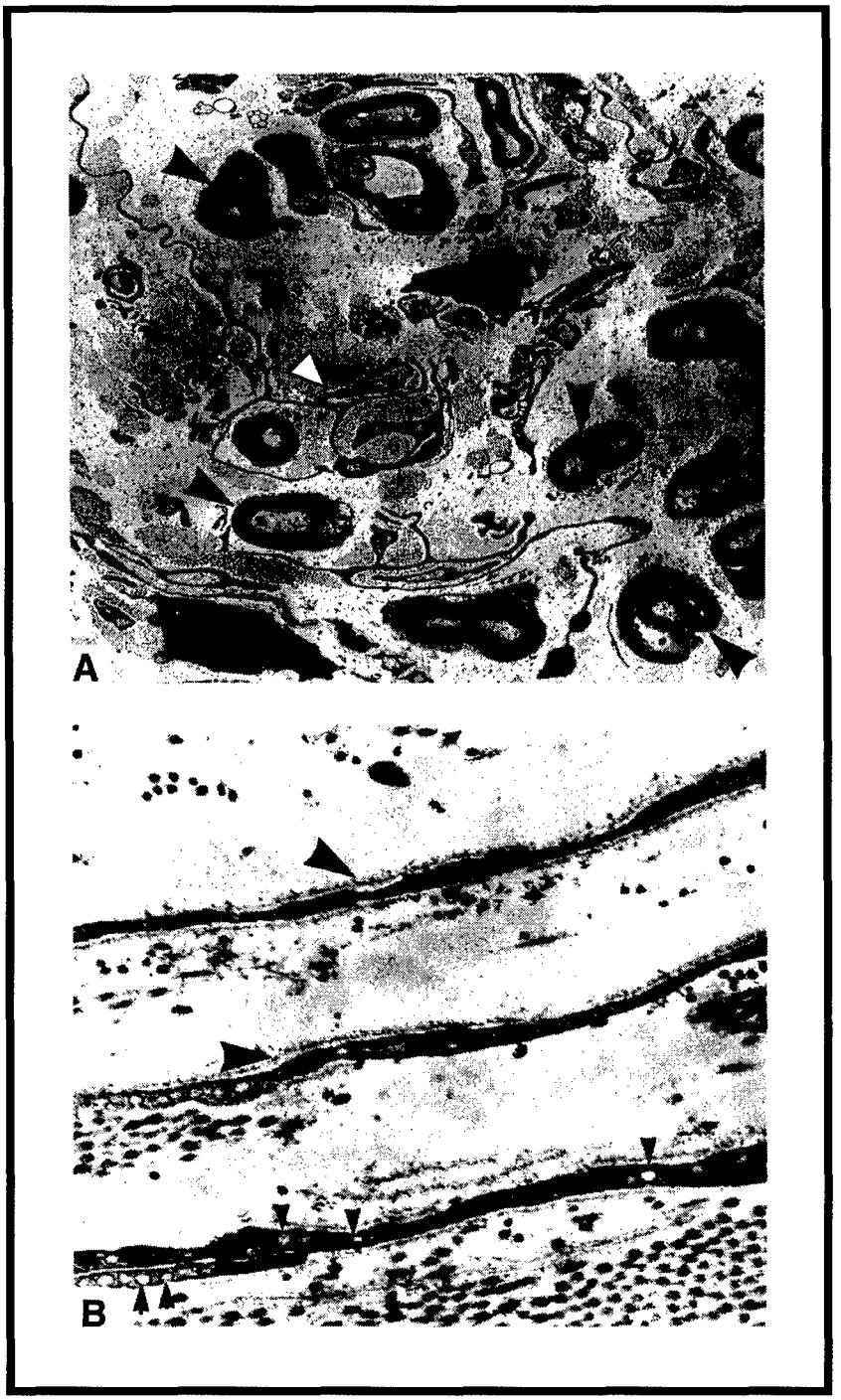

Figure 2. EM analysis of tumors from Nf1-/- chimeric mice.
A) Closed arrowheads illustrate the presence of normal myelenated nerves in this tumor. The open arrowhead is pointing to one of the many cells that exhibit extensive branching: a unique characteristic of Schwann cells. B) A magnified view of a tumor showing cells with basal lamina, which is indicative of Schwann and/or perineurial cells. The top arrow is pointing to a cell which is most closely resembles a Schwann cell since it contains an uninterrupted basal lamina and very few pinocytotic vessicles. The bottom two arrows point to cells which appear to have a discontinuous basal lamina and multiple pinocytotic vessicles (samller arrows) and therefore suggests perineurial of perineurial/Schwann-likecells (suggested to represent a putative transitional cell). Each of these cell types have been previously observed in human neurofibromas.

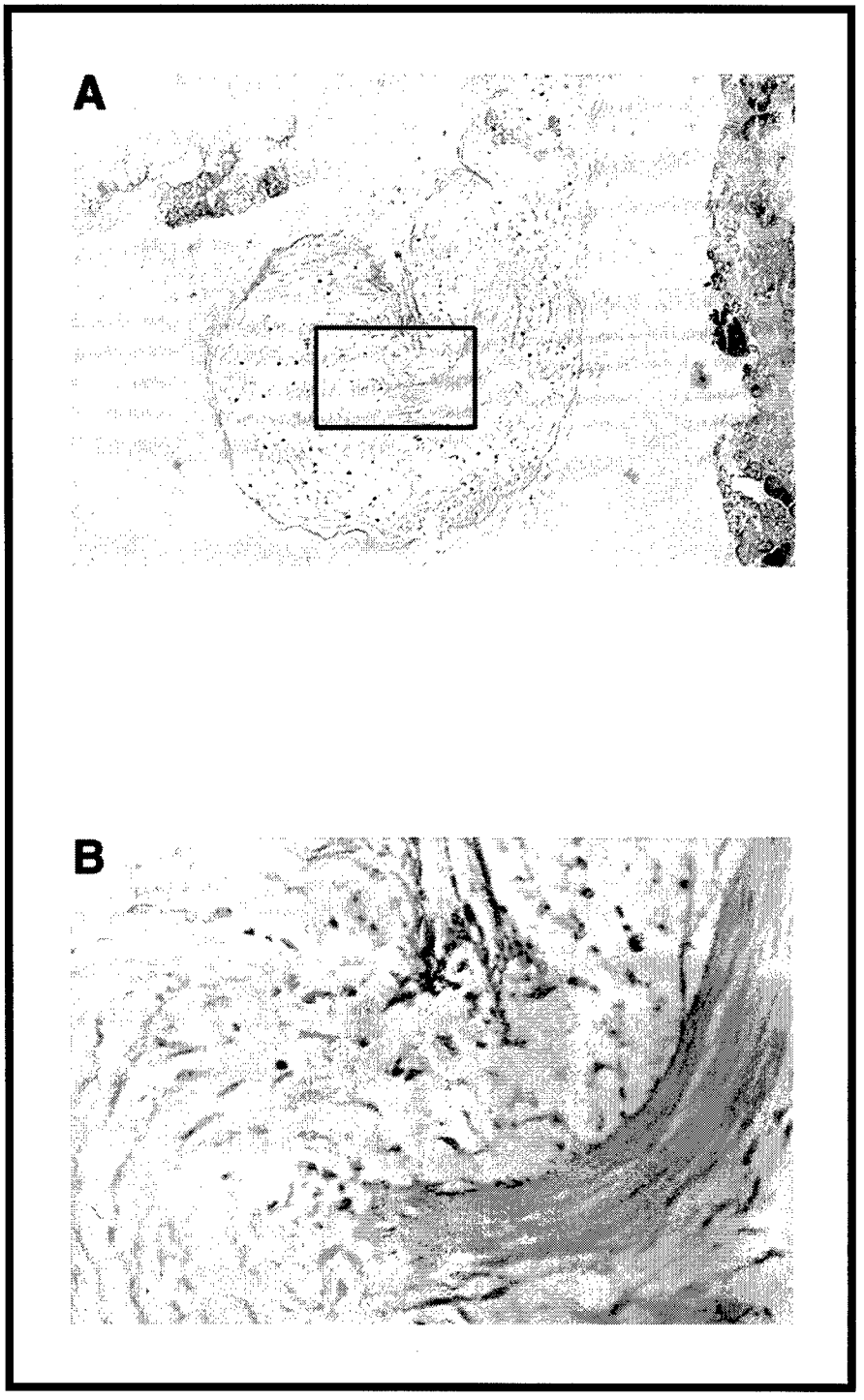

Figure 3. B-galactosidase expression of tumors from Nf1 -/-, lacZ+ chimeric mice. A) 10x magnification of a tumor containing a normal central nerve (longitudinal section) and surrounding tissue. Note the patchy staining in the outlaying non-tumorigenic regions in contrast to the homogeneous staining of the tumor. B) 40x magnification of this tumor more clearly demonstrates ubiquitous B-galactosidase expression.

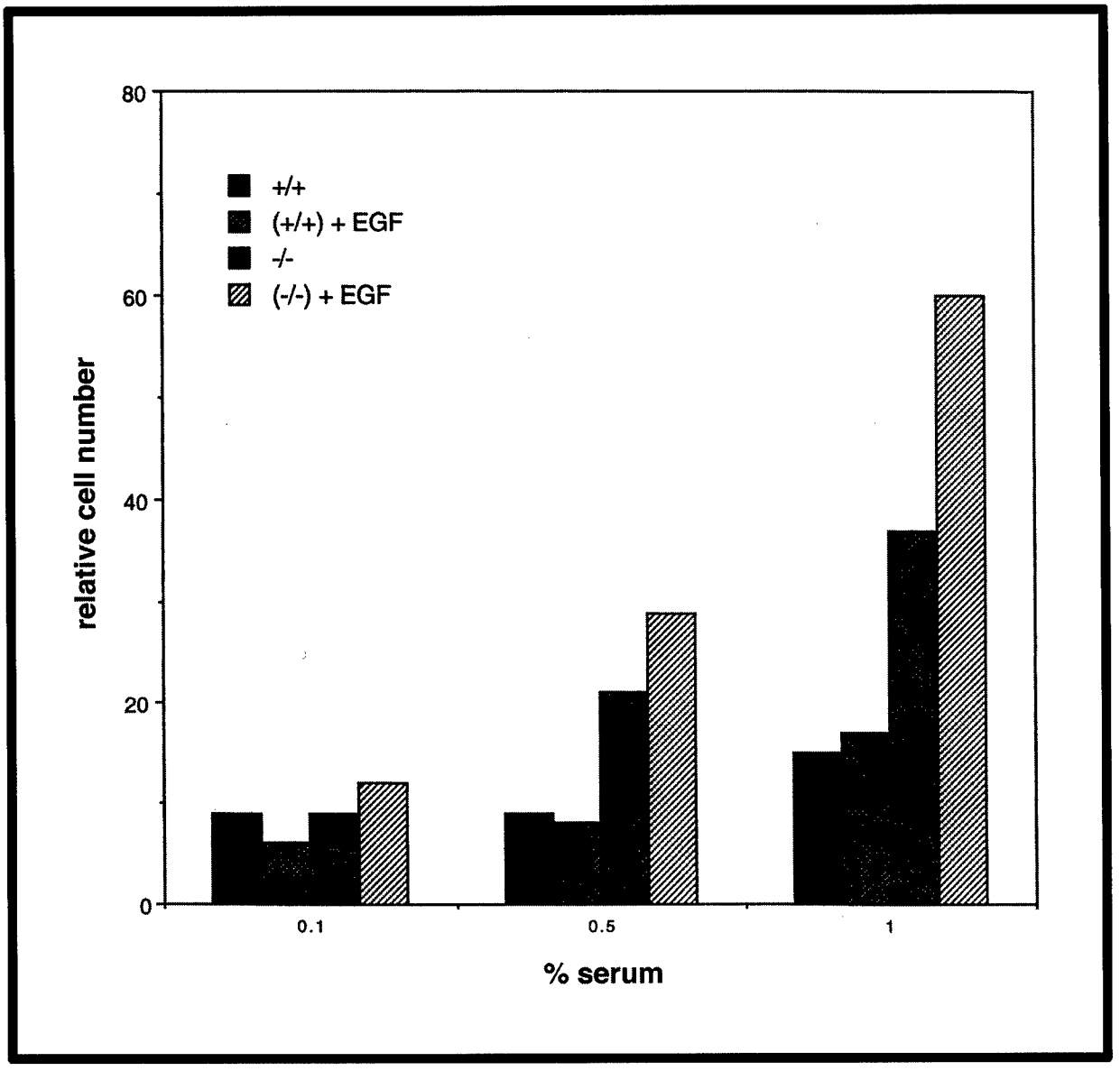

Figure 4. Nf1-/- MEFs are hypersensitive to serum and EGF. Wild type and Nf1-/- MEFs were grown to confluency and serum starved. The cells were then split and grown in increasing concentrations of serum and/or EGF. Cell number was determined six days after replating.

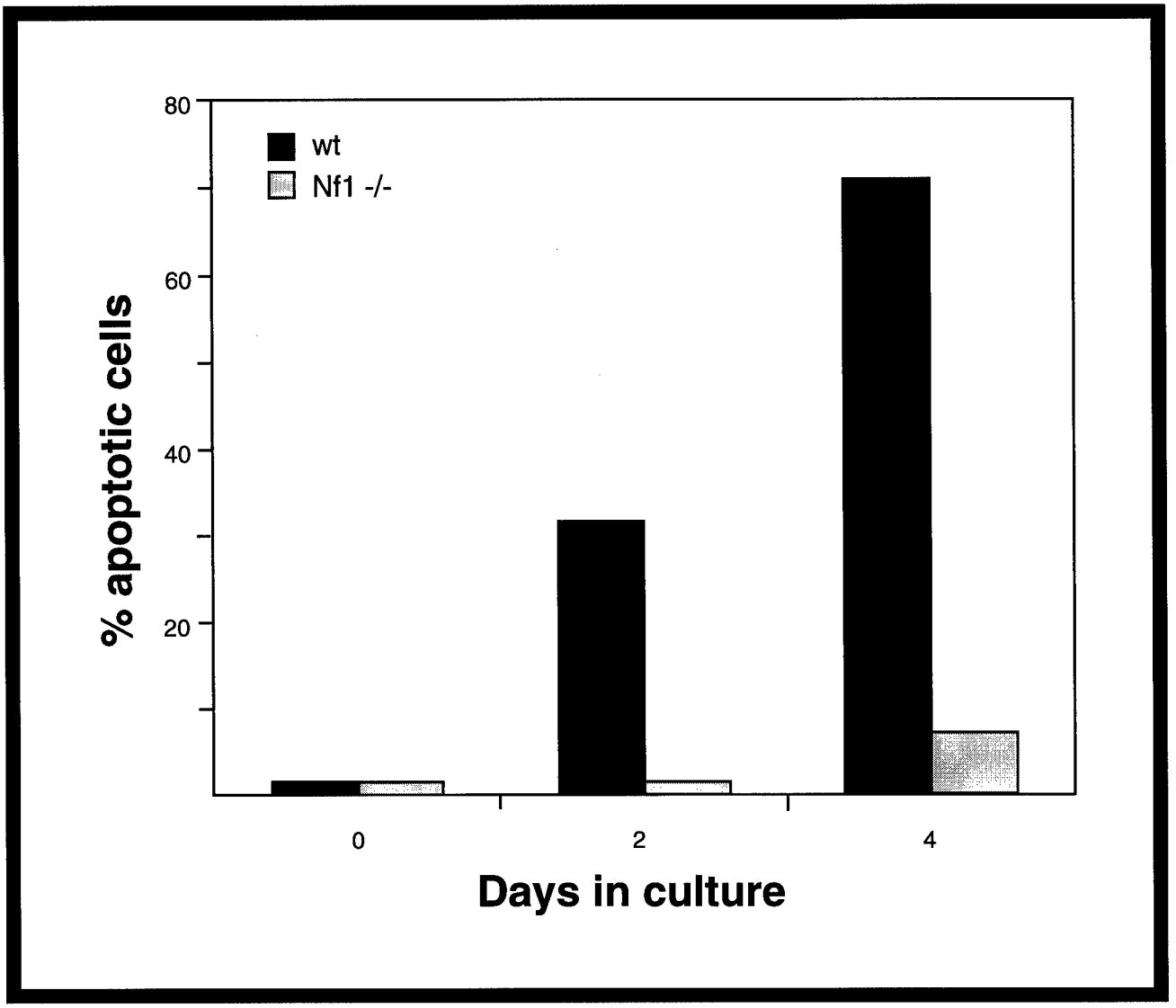

Figure 5. *Nf1* -/- MEFs do not undergo apoptosis following serum withdrawl. MEFs were trypainized, repeatedly washed in serum free media and plated at a moderate cell density. Dead cells were quantitated by trypan blue exclusion and further examined by TUNEL analysis.

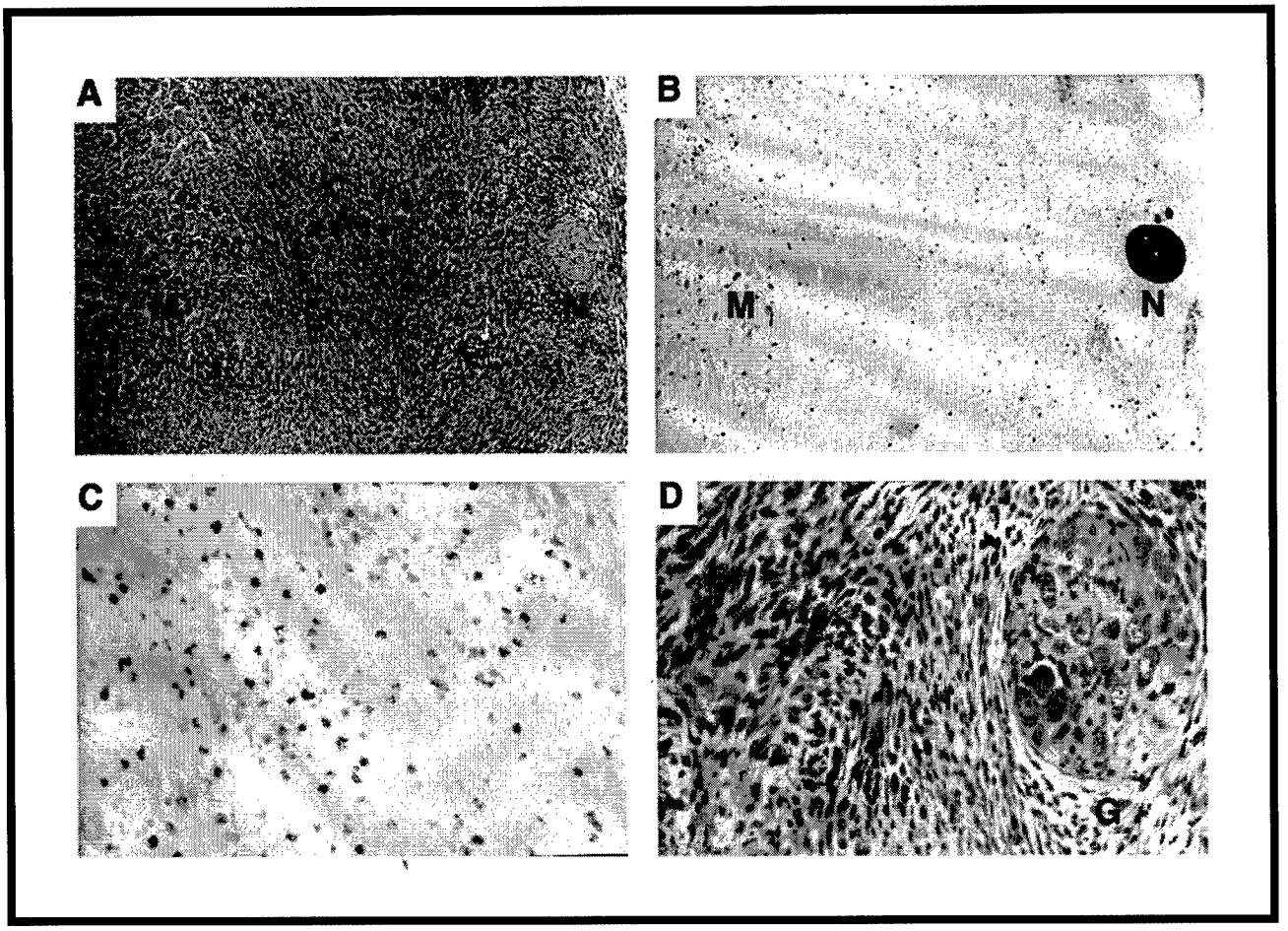

Figure 6. Histological analysis of cis mice. A-10x and D-40x) Hematoxylin and eosin stained tumors are histologically identical to MPNST's which typically invade muscle (A, left) contain peripheral nerves (A and D, right) and contain cells with spindle-shaped and/or wavy nuclei. B-10x and C-40x) S100 staining of the same tumor showing the positivity of cells associated with the normal nerve (N) and throughout the tumor.

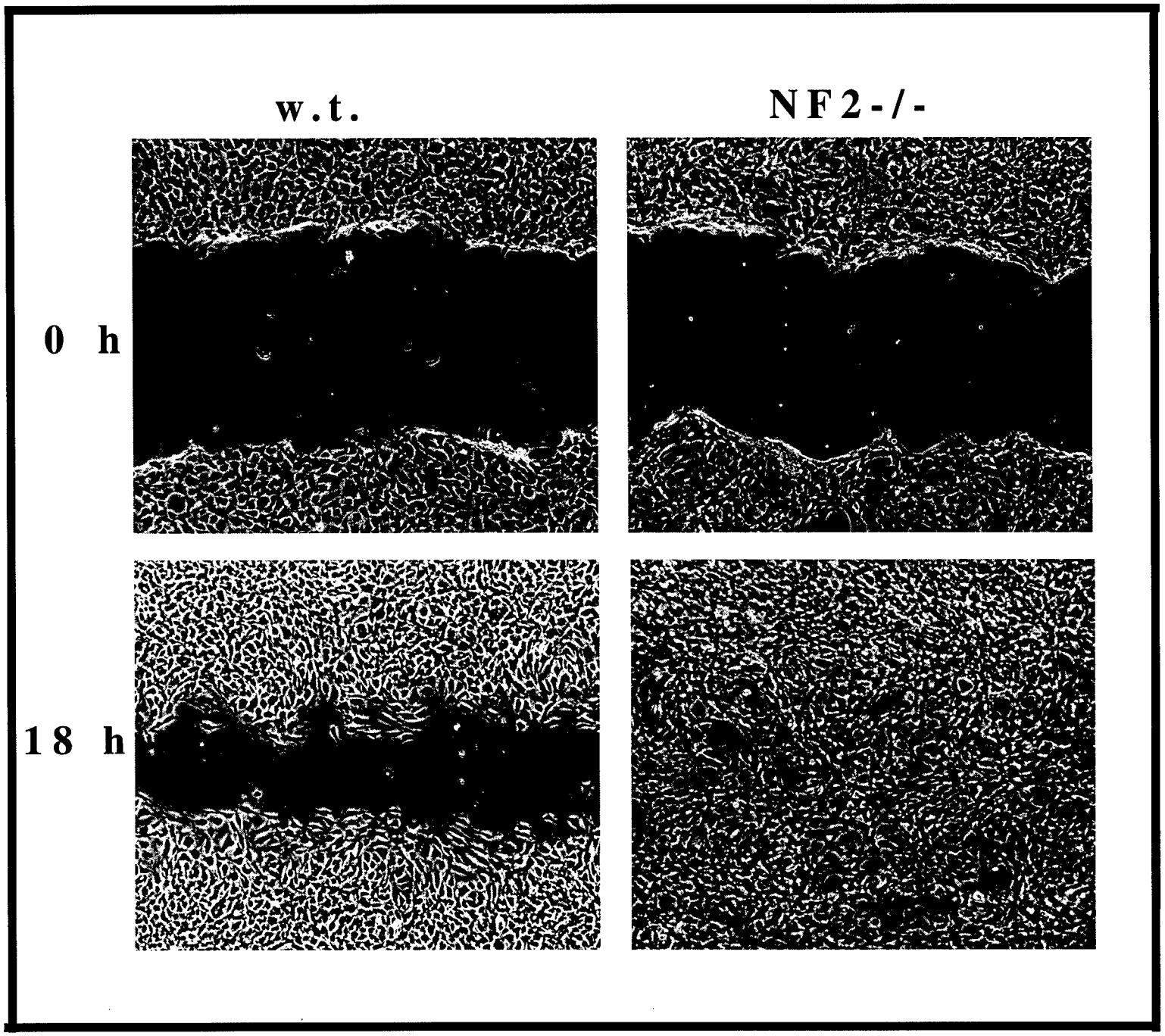

Figure 7. NF2-deficient fibroblasts wound heal 2-3 times faster than their wild-type counterparts. Wild-type and NF2-deficient mouse embryo fibroblasts were plated in full serum and then mechanically wounded and followed for 24h after wounding by phase contrast microscopy. Despite sharing similar growth properties in full serum, the Nf2 deficient cells migrated into the wound 2-3 times faster than the wild-type cells.

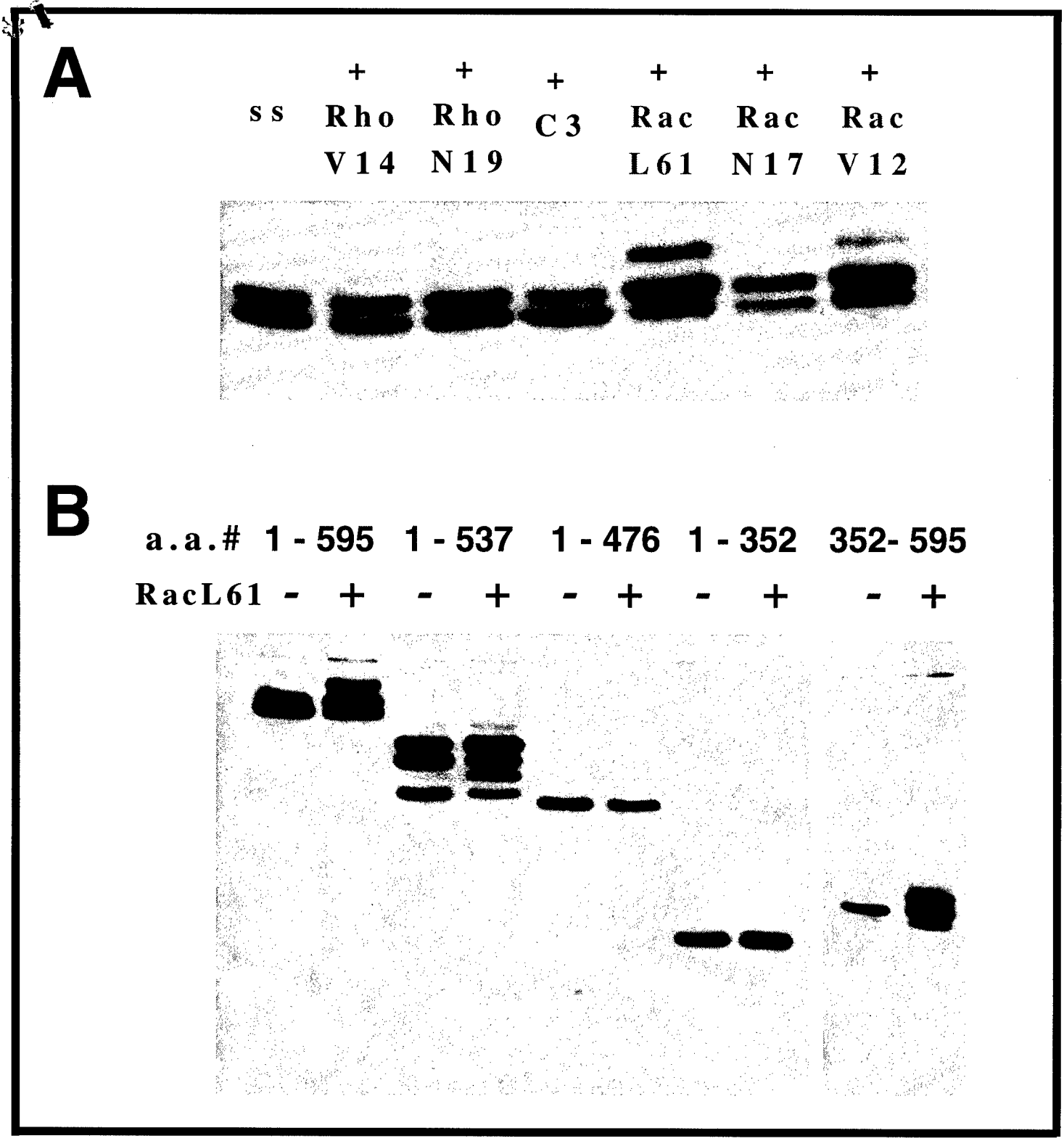

Figure 8. Activated alleles of the Rac GTPase, not Rho, result in hyperphosphorylation of merlin's carboxyl terminal residues. *A*) Merlin was co-transfected with activated (V14, L61, V12) alleles or dominant negative (N19, C3, N17) alleles of RhoA and Rac1 GTPase in NIH3T3 cells. Merlin's phosphorylation was detected by immunoblotting for merlin. While activated RhoA has no effect on merlin's phosphorylation state, two different activated alleles of Rac induced potent phosphorylation of the protein. B) Epitope tagged deletion mutants were tranfected with and without activated Rac (as indicated by +). Phosphorylation is detected by bandshift in immunoblot against epitope tag. A Rac-responsive phosphorylation site resides between amino acids 476 to 537.

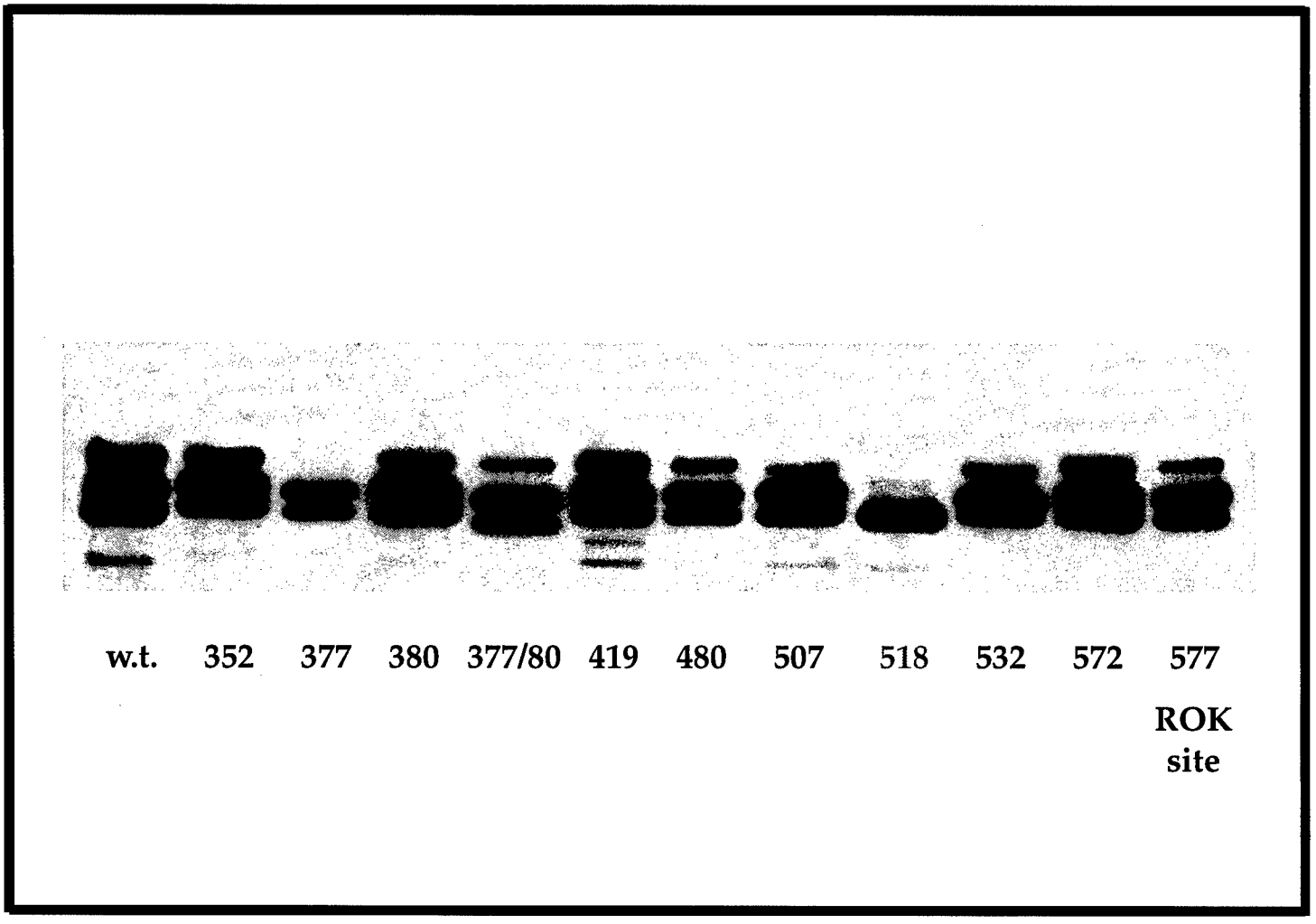

Figure 9. Mutation of Merlin Serine 518 to Alanine abolishes Rac-dependent Phosphorylation. Sire-directed mutagenesis was used to mutate individual serine or threonine residues in the carboxyl terminus of merlin that bore kinase consensus motifs. Mutated residue is indicated by amino acid number. Cells were then transiently transfected with each alanine mutant along with an activated allele of Rac. Phoshorylation was detected by immunoblotting for transfected merlin.

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FOR THE COMMANDER:

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